Marine Biology 22, 353—354 (1973)

In vitro Fertilization with Non-Motile Spermatozoa of the Brown Shrimp *Penaeus aztecus*

W. H. Clark, Jr.[1], P. Talbot[1], R. A. Neal[2], C. R. Mock[2] and B. R. Salser[2]

[1] Department of Biology, University of Houston; Houston, Texas, USA
and
[2] National Marine Fisheries Service, Biological Laboratory; Galveston, Texas, USA

Abstract

The sperm of the brown shrimp *Penaeus aztecus* are non-flagellated and non-motile. *In vitro* fertilization can be obtained when mature eggs removed from female shrimp are added to dense sperm suspensions in seawater and agitated in a manner simulating natural spawning.

Introduction

Most sperm in the animal kingdom are flagellated, with notable exceptions occurring among the Nematoda and Arthropoda. Techniques for successful *in vitro* fertilization using flagellated sperm have been reported for numerous invertebrates (Costello *et al.*, 1957) and vertebrates (Austin, 1972) but, to our knowledge, such success has never been realized for animals possessing non-motile sperm. Previous reports based on light microscopy have stated that the sperm of penaeid shrimp have "tails" (Hudinaga, 1942; King, 1948) and appear motile (King, 1948). In this study, we present electron microscopical evidence for a non-flagellated penaeid sperm, and we describe a technique for *in vitro* fertilization with gametes of the penaeid shrimp *Penaeus aztecus*. To our knowledge, this is the first time *in vitro* fertilization has been successfully accomplished with non-motile spermatozoa. This technique will enable investigators to more fully understand the events of fertilization in higher crustaceans. In addition, it will be extremely useful to the mariculture industry in the experimental breeding and possibly in the hybridization of the penaeid shrimp.

Materials, Methods and Results

Sperm Structure

Brown shrimp, *Penaeus aztecus*, were collected from the Gulf of Mexico off Freeport, Texas, USA. Sperm were taken from the male reproductive tract and examined with phase microscopy. For deoxyribonucleic acid (DNA) localization, acridine orange, a fluorochroming agent, was used (Clark *et al.*, 1972). For electron microscopy, sperm were pre-fixed in half-strength Karnovsky's fixative (Karnovsky, 1965), postfixed in phosphate-buffered 1% osmium tetroxide, dehydrated in acetone, and embedded in a low viscosity plastic (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate and examined using an AEI EM-6B or Hitachi HS-8F-2 electron microscope.

Under the phase microscope, the sperm appeared to be composed of a spherical body and a small "spike". These sperm exhibited no motility. With the electron microscope, the spherical body and "spike" are seen in detail (Fig. 1A). Centrally located in the body of the sperm is a large, non-membrane bound, nucleoid-like region which contains DNA as determined with acridine orange. This region is composed of a mesh-like network of fibrils and electron-dense accumulations of flocculent material. Between the nucleoid-like region and the plasma membrane is a cytoplasmic band containing concentric whorls of membranous material and two distinct species of vesicles. Subjacent to and extending laterally from the base of the "spike" the cytoplasmic band appears granular and lacks the components previously described. The "spike" extends 2.5 μ from the body of the sperm. It is narrow at its distal end and progressively widens to form a cup-shaped base which is closely apposed to the granular cytoplasmic region. The "spike" contains no substructural elements (microtubules, microfilaments, or filaments) which might be related to contractility or mobility[1]. Instead, the "spike" is composed of an amorphous, electron-dense material (Fig. 1B). Surrounding and extending away from the base of the "spike" is an amorphous region of low electron density which terminates at the boundary between the granular and organelle-containing cytoplasmic bands.

On the basis of both our phase and electron microscopic observations, we concluded that the sperm of *Penaeus aztecus* is indeed non-flagellated and non-motile. This explained, in part, our unsuccessful attempts with *in vitro* fertilization in the past (the simple

[1] Brown (1967) has described cross-banded fibers in the "spikes" of spermatozoa from the shrimp, *Palaemonetes paludosus*. No function has been ascribed to these structures.

Fig. 1. *Penaeus aztecus*. (A) Electron micrograph of shrimp sperm. Body of sperm contains a central nucleoid-like region (*N*). Bounding the nuclear material is a cytoplasmic band containing membranous material, small vesicles (small arrows), and larger vesicles with a fibrillar matrix (large arrow). The portion of the cytoplasmic band subjacent to the "spike" (*S*) contains only granular material (*G*). Extending laterally from the base of the "spike" is a zone of amorphous material of low electron density (*A*) (× 17,900). (B) Tangential section through the "spike" (×39,000)

mixing of sperm and eggs in seawater). Thus, to determine how the spermatozoa come in contact with eggs, natural spawning was observed.

In vitro Fertilization

During natural spawning the female appears to release eggs and seminal plasm containing sperm simultaneously, vigorously mixing the two with her pleopods. In developing a technique for fertilization, we have attempted to duplicate this mixing action. Ampoules (seminal vesicles) were removed from sexually mature males (130 to 150 mm in length) and macerated in seawater to form a viscous sperm suspension (54,000 sperm/ml sea water). Gravid females were dissected and their ovaries removed. The ovaries were placed in seawater and teased into small pieces. These teased portions of ovary were agitated in seawater to release free ova. Egg samples (approximately 1000 free eggs in a small aliquot of seawater) were pipetted into 500 ml Ehrlenmeyer flasks, each containing 100 ml of sperm suspension. The flasks were moved rapidly in a gyratory path for approximately 5 min. The sperm-egg suspensions were then decanted into inverted 5 gallon (18.9 l) bell jars containing seawater. The salinity and pH of the seawater were 31‰ and 8.1, respectively. The seawater in the bell jars was maintained at a temperature of 29 °C, and vigorously aerated. Eggs containing nauplii stages were noted 7 h post *in vitro* fertilization. Approximately 10% of the initial eggs hatched and developed normally through nauplius, protozoea, mysis and post-larval stages. At the time of submission of this paper, the postlarvae are 50 mm in length.

Other egg samples were obtained and treated as described above, but were not mixed with sperm. These samples were placed in bell jars under the same conditions as the fertilized eggs. They exhibited no development.

Summary

1. Fine structural studies and phase-microscopic observations on sperm of the penaeid shrimp *Penaeus aztecus* have indicated that the sperm is non-flagellated and non-motile.

2. A technique is described which has been successfully used to fertilize penaeid ova *in vitro*.

Literature Cited

Austin, C. R.: Fertilization. *In:* Reproduction in mammals. I. Germ cells and fertilization, pp 103—133. Ed. by C. R. Austin and R. V. Short. London: Cambridge Press 1972.

Brown, G. C.: Gamete surface components. *In:* Fertilization, Ed. by C .B. Metz and A. Monroy. New York: Academic Press 1967.

Clark, W. H. Jr., R. L. Moretti and W. W. Thomson: Histochemical and ultracytochemical studies of the spermatids and sperm of *Ascaris lumbricoides* var. *suum*. Biol. Reprod. **7**, 145—159 (1972).

Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox and C. Henley: Methods for obtaining and handling marine eggs and embryos, 247 pp. United States: Lancaster Press 1957.

Hudinaga, N.: Reproduction, development and rearing of *Penaeus japonicus* Bate. Jap. J. Zool. **10**, 305—393 (1942).

Karnovsky, M. J.: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. **27**, 137A—138A (1965).

King, J. E.: A study of the reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linneaus). Biol. Bull. mar. biol. Lab., Woods Hole **94**, 244—262 (1948).

Spurr, A. R.: A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**, 31—43 (1969).

First author's address: Dr. W. H. Clark, Jr.
Department of Biology
University of Houston
Houston, Texas 77004
USA

Date of final manuscript acceptance: August 3, 1973. Communicated by J. Bunt, Miami